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Developmental Biology:
Molecular and Therapeutic
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Molecular and Therapeutic Characterization of Anti-ectodysplasin A Receptor (EDAR) Agonist Monoclonal Antibodies^{*[5]}

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The TNF family ligand ectodysplasin A (EDA) and its receptor EDAR are required for proper development of skin appendages such as hair, teeth, and eccrine sweat glands. Loss of function mutations in the *Eda* gene cause X-linked hypohidrotic ectodermal dysplasia (XLHED), a condition that can be ameliorated in mice and dogs by timely administration of recombinant EDA. In this study, several agonist anti-EDAR monoclonal antibodies were generated that cross-react with the extracellular domains of human, dog, rat, mouse, and chicken EDAR. Their half-life in adult mice was about 11 days. They induced tail hair and sweat gland formation when administered to newborn EDA-deficient *Tabby* mice, with an EC₅₀ of 0.1 to 0.7 mg/kg. Divalency was necessary and sufficient for this therapeutic activity. Only some antibodies were also agonists in an *in vitro* surrogate activity assay based on the activation of the apoptotic Fas pathway. Activity in this assay correlated with small dissociation constants. When administered *in utero* in mice or at birth in dogs, agonist antibodies reverted several ectodermal dysplasia features, including tooth morphology. These antibodies are therefore predicted to efficiently trigger EDAR signaling in many vertebrate species and will be particularly suited for long term treatments.

The TNF family includes 19 ligands, most of which control development, function and/or homeostasis of the immune sys-

tem (1). In this respect, ectodysplasin A (EDA)⁸ is an exception as it participates in ectodermal appendage formation (2). The *Eda* gene on the X chromosome is transcribed as multiple splice variants, only two of which code for the receptor-binding C-terminal TNF homology domain. These two variants, generated by splicing at an alternative donor site between exons 8 and 9, code for 391- and 389-amino acid-long proteins called EDA1 and EDA2 (3). EDA1 binds EDAR, whereas EDA2 binds to another receptor, XEDAR (3). The biology of EDA2 and XEDAR is distinct from that of EDA1. Indeed, XEDAR-deficient mice have no obvious ectodermal dysplasia phenotype, whereas mice deficient in EDA, EDAR, or the signaling adaptor protein EDARADD all display virtually indistinguishable ectodermal dysplasia phenotypes, indicating the predominance of the EDA1-EDAR axis in the development of skin-derived appendages (4–8).

In humans, EDA1 loss of function mutations cause X-linked hypohidrotic ectodermal dysplasia (XLHED), a rare condition characterized by defective formation of teeth, hair, sweat glands and other glands (6). Because of their insufficient number of sweat glands, these patients are prone to hyperthermia. They also frequently suffer from recurrent respiratory tract infections caused by abnormal mucus production in the airways. Other problems are oligodontia, dry skin, and dry eyes (9–11).

EDA1 is a transmembrane type II protein with a furin consensus cleavage site, a collagen-like domain, and a C-terminal TNF homology domain, any of which when mutated can cause XLHED (12). To be active, EDA must be processed and bind EDAR through its trimeric C-terminal domain. The signaling ability of EDA1 is re-enforced by its collagen domain that cross-links individual EDA1 trimers (13). Interestingly, some EDA1 mutations can also cause selective tooth agenesis, a condition characterized by no or very little involvement of other ectodermal appendages (14). In these patients, EDA1 mutants retain partial binding to EDAR, suggesting that tooth development is particularly sensitive to “high quality” EDAR signals.

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S8.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) JN099705 to JN099733.

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⁸ The abbreviations used are: EDA, ectodysplasin A; EDAR, EDA receptor; XLHED, X-linked hypohidrotic ectodermal dysplasia.

Transgenic expression of EDA1 in skin under the keratin 14 promoter results in a disheveled hair phenotype, hypertrophy of sebaceous glands, and formation of supernumerary molars or nipples (15). Transgenic EDA1 expression in the skin of EDA-deficient *Tabby* mice corrected many of the ectodermal dysplasia defects (16). The reverted phenotype was stable even after shutdown of transgenic EDA1 expression in young adults, suggesting that EDA1 plays a role in the formation but not in the maintenance of skin appendages. Interruption of EDA1 expression, however, resulted in the normalization of sebaceous gland size (16). Similar conclusions were reached with an alternative approach of protein replacement therapy, in which EDA-deficient animals were exposed to a recombinant form of EDA during development (17, 18). Taken together, these data provide a proof of concept for protein replacement therapy in young patients with XLHED.

In this study, we generated agonist anti-EDAR antibodies that mimic the action of transgenic or recombinant EDA1 in development. Most of these antibodies cross-react with EDAR of mammals and birds and are active as monomeric, divalent molecules. They corrected, among others, sweat glands, tracheal glands, and tooth morphology in EDA-deficient mice and were also active in EDA-deficient dogs. These mouse monoclonal antibodies will be reagents of choice for long term experiments in mice and pave the way for the development of therapeutic antibodies for use in XLHED or other EDAR-related applications in humans.

EXPERIMENTAL PROCEDURES

Animals—Mice were handled according to Swiss Federal Veterinary Office guidelines, under the authorization of the Office Vétérinaire Cantonal du Danton de Vaud (authorization 1370.3 to P. S.). White-bellied agouti B6CBAa $A^{w-J}/A-Eda^{Ta}/Tabby$ mice (000314; The Jackson Laboratory) were bred as Eda^{Ta}/Eda^{Ta} and Eda^{Ta}/Y mutants or as $+/+$ and $+/Y$ wild type controls. EDAR-deficient OVE1B mice were as described previously (5). EDA-deficient dogs (19) were cared for in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and in the International Guiding Principles for Biomedical Research Involving Animals.

Plasmids and Recombinant Proteins—Plasmids used in this study were either previously published or derived from the published plasmids by standard molecular biology techniques (supplemental Fig. S1) (13, 20, 21). A fully human form of Fc-EDA1 was kindly provided by Dr. Neil Kirby (EdimerPharma, Boston). hEDAR-Fc and mEDAR-Fc were produced and purified as described previously (21).

Generation and Purification of Anti-EDAR Monoclonal Antibodies—150 μ g of hEDAR-Fc or mEDAR-Fc (amino acid residues 29–183 supplemental Fig. S1) were briefly sonicated three times in 750 μ l of PBS/STIMUNE (1:1, v/v) (Cedi-diagnostics, Lelystad, The Netherlands). Female OVE1B mice were immunized subcutaneously (base of tail, 200 μ l) with the antigen preparation and boosted between days 10 and 14 with antigen in PBS/STIMUNE (base of tail, 150 μ l). Mice positive for anti-EDAR antibodies at day 30 were boosted with 150 μ g of antigen in PBS at day 40 (base of tail). Three days later, lymph

node cells were fused with myeloma cells according to standard procedures, grown in complete RPMI 1640 medium over a feeder layer of mouse macrophages, and selected 24 h later with hypoxanthine/aminopterin/thymidine-containing medium. Supernatants of 96-well plates were tested by ELISA for antibody secretion. Positive clones were subcloned twice by limiting dilution and then slowly adapted to medium without macrophages and hypoxanthine/aminopterin/thymidine medium supplement. Most hybridomas could then be progressively adapted to serum-free Opti-MEM medium (Invitrogen). Antibodies were purified from conditioned Opti-MEM supernatants by affinity chromatography on protein G-Sepharose (GE Healthcare).

Transfections—HEK 293T cells were grown in DMEM, 10% fetal calf serum and transfected by the calcium phosphate method. Cells were grown for 7 days in serum-free Opti-MEM medium (Invitrogen) for the production of EDAR-Fc truncation mutant fusion proteins or for 48 h in complete medium for surface expression of receptors-TRAILR3 fusion proteins.

ELISA—For the detection of anti-EDAR antibodies, ELISA plates were coated with hEDAR-Fc at 1 μ g/ml, blocked, and revealed with anti-EDAR antibodies (adequately diluted serum of EDAR immunized mice, hybridoma supernatants, or purified antibody) followed by a peroxidase-coupled goat anti-mouse IgG (Jackson ImmunoResearch). For isotype determination, ELISA plates were coated with 1 μ g/ml of anti-EDAR antibodies and revealed with peroxidase-coupled antibodies against the heavy chain of mouse IgG1, IgG2a, or IgG2b (Southern Biotech). For epitope mapping, ELISA plates were coated with an F(ab')₂ fragment of a goat anti-human Ig (Jackson ImmunoResearch) to capture various EDAR-Fc constructs or a control receptor (B cell maturation antigen-Fc) in cell supernatants. EDAR-Fc constructs and B cell maturation antigen-Fc were revealed either with peroxidase-coupled donkey anti-human (H+L) (Jackson ImmunoResearch) or with anti-EDAR antibodies at 1 μ g/ml followed by peroxidase-coupled goat anti-mouse IgG (Jackson ImmunoResearch) or with FLAG-EDA1 or FLAG-BAFF followed by biotinylated anti-FLAG M2 antibody (Sigma) and peroxidase-coupled streptavidin.

SDS-PAGE, Western Blot, and Native Gel Electrophoresis—Anti-EDAR antibodies (10 μ g/lane) were analyzed by SDS-PAGE under reducing conditions followed by Coomassie Blue staining. Antibodies were also analyzed by native gel electrophoresis (4 μ g/lane) (Biomidi, Toulouse, France) and stained with Amido Black according to manufacturer's instructions, except that the electrophoresis was performed for 1 h.

To test the ability of anti-EDAR antibodies to recognize denatured EDAR, 2 μ g of bovine serum albumin, 20 ng of hEDAR-Fc, and 20 ng of hFas-Fc were analyzed by SDS-PAGE and Western blot under reducing (100 mM dithiothreitol) or nonreducing conditions, revealed with the various anti-EDAR antibodies at 1 μ g/ml followed by peroxidase-coupled anti-mouse antibody (1:10,000) and ECL reagent (GE Healthcare).

Sequencing of Anti-EDAR Antibodies—RNA was extracted from hybridoma cells with an RNeasy kit (Qiagen) according to the manufacturer's guidelines. cDNA was prepared by reverse transcription with Ready-To-Go T-Primed first-Strand kit (GE Healthcare). Variable sequences of the heavy

and light chains were amplified by PCR as described previously (22). PCR products were sequenced on both strands. Sequences were analyzed for gene usage using the IMGT sequence alignment software.

FACS Analyses—293T cells co-transfected with enhanced GFP, and receptor-glycosylphosphatidylinositol expression plasmids were stained with Fc-EDA1 or Fc-EDA2 essentially as described before (20) or with anti-EDAR antibodies at 4 μ g/ml followed by phycoerythrin-coupled anti-mouse secondary antibody. Following staining, cells were analyzed using a FACScan flow cytometer (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

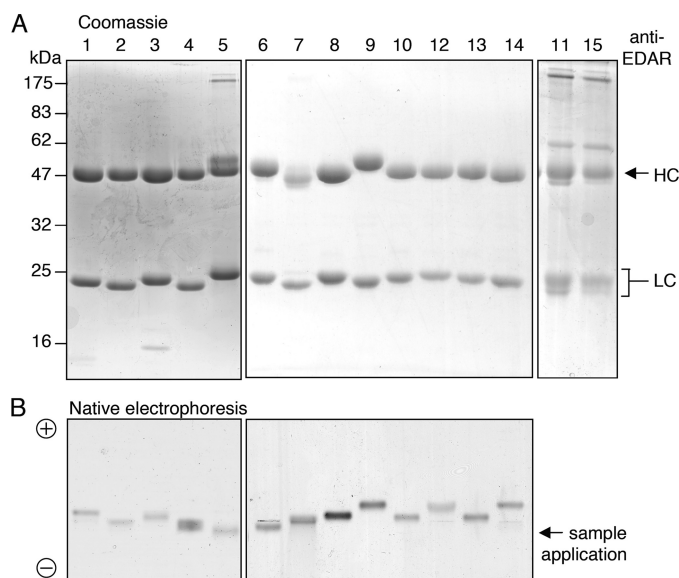


FIGURE 1. Purity of protein-G purified anti-EDAR monoclonal antibodies. Anti-EDAR monoclonal antibodies were purified by protein G affinity chromatography from culture supernatants in serum-free medium (antibodies 1–4, 6–10, and 12–14) or in serum-containing medium (antibodies 5, 11, and 15). **A**, SDS-PAGE analysis and Coomassie Blue staining of 10 μ g of antibody per lane under reducing conditions. Migration positions of molecular mass standards (in kDa) are shown. **B**, native gel electrophoresis of 4 μ g of anti-EDAR antibodies per lane, stained with Amido Black.

TABLE 1

Characteristics of anti-EDAR monoclonal antibodies

IGHV and IGLV indicate immunoglobulin heavy and light chain variable region genes likely used in the antibody. hu is human EDAR-Fc; mu is mouse EDAR-Fc. I + II indicates EDAR-Fc containing CRD1 and CRD2 of human EDAR. I is EDAR-Fc containing CRD1 only. Full is EDAR-Fc containing the full extracellular domain of EDAR. k_a is the association constant. k_d is the dissociation constant. K_D is the affinity of monomeric Fab to EDAR-Fc. EC₅₀ tail hair indicates the dose of antibody required to get half-maximal tail hair reversion score when administered intraperitoneally in newborn *Tabby* mice. EC₅₀, mEDAR:Fas or hEDAR:Fas means dose of antibody required to kill half of the EDAR:Fas-expressing Jurkat cells.

Anti-EDAR	IGHV gene	IGLV gene	Antigen	Isotype	Epitope	$k_a \cdot 10^{-5}$ $M^{-1} s^{-1}$	$k_d \cdot 10^4$ s^{-1}	K_D nM	EC ₅₀		
									Tail hair mg/kg	mEDAR:Fas ng/ml	hEDAR:Fas ng/ml
1	5–17	10–96	hu	IgG1	I + II	6.79	3.69	0.54	0.125	10	200
2	1–69	1–117	hu	IgG1	I + II	13.2	13.3	1.00	0.42	>4000	>4000
3	5–17	10–96	hu	IgG1	I + II	ND	ND	ND	0.18	30	100
4	ND	1–117	hu	IgG1	I + II	4.50	13.6	3.01	0.7	>4000	>4000
5	2–2	4–91	hu	IgG2b	I	ND	ND	ND	0.25	>4000	>4000
6	1S135	1–110	hu	IgG2a	I	3.81	6.02	1.58	0.25	>4000	>4000
7	7–3	4–577	hu	IgG1	I + II	2.92	96.6	33.1	0.3	>4000	>4000
8	5–17	10–96	mu	IgG1	I + II	8.12	3.86	0.48	0.5	100	>4000
9	1–63	1–117	mu	IgG1	I+II+III	1.89	8.44	4.46	0.42	>4000	>4000
10	1–39	4–55	hu	IgG1	I + II	0.35	2.38	6.78	0.125	10	50
11	1–39	10–94	hu	IgG1	I + II	ND	ND	ND	3.3	>4000	>4000
12	1–14	17–12	mu	IgG1	I	0.41	6.66	16.3	0.35	10	5
13	1S135	1–110	hu	IgG1	I	1.83	18.7	10.2	0.42	>4000	>4000
14	1–42	10–94	mu	IgG1	I + II	1.90	74.8	39.3	0.3	>4000	>4000
15	1–63	12–44	hu	IgG1	I + II	ND	ND	ND	ND	ND	ND

Fab Generation and Affinity Determination by Surface Plasmon Resonance—An IgG1 Fab and F(ab')₂ preparation kit was used according to the manufacturer's instructions (Pierce). Briefly, purified anti-EDAR antibodies were digested for 72 h at 37 °C with immobilized ficin. Fc fragments and undigested antibodies were removed by chromatography on protein A. The flow-through, containing the Fab and F(ab')₂ fragments, was concentrated and applied onto a Superdex-200 gel permeation chromatography column eluted in PBS. Absorbance was recorded at 280 nm.

For surface plasmon resonance, human EDAR-Fc was captured on anti-human IgG Fc-derivatized CM5 chips in a Biacore T100 apparatus (GE Healthcare). Fab solutions of anti-EDAR antibodies at the indicated concentration in PBS were applied for 90 s at 50 μ l/min and subsequently washed with buffer. All curve fittings were performed assuming a 1:1 binding model, although two antibodies had a biphasic dissociation that did not fit the 1:1 model.

In Vitro Cytotoxicity Assays—Fas-deficient hEDAR:Fas Jurkat cells have been described before, and mEDAR:Fas-expressing cells were obtained by retroviral infection according to the same protocol (13). The cytotoxicity assay using EDAR:Fas Jurkat cells was performed as described for FasL on Jurkat cells (23).

Injections in EDA-deficient Animals—*Tabby* pups were labeled by puncture of a footpad with a 30-gauge needle dipped in china ink. Intraperitoneal injections with anti-EDAR antibodies or Fc-EDA1 were performed within 24 h after birth with a maximal volume of 15 μ l, using 0.5-ml U-100 insulin syringes (BD Biosciences). Examination and photography of tail hairs were performed at days 20–22 post-injection. Pregnant *Tabby* mice were treated intravenously at days 13 and 20 (E13/E20) or 9 and 17 (E9/E17) of gestation with 400 μ g of anti-EDAR antibody (antibody 3). Offspring were analyzed at 6 months of age, essentially as described previously (18). Age-matched wild type and EDA-deficient *Tabby* mice were similarly analyzed for comparison. Tracheal glands were detected by Alcian blue staining (24). Three dogs affected with X-linked ectodermal

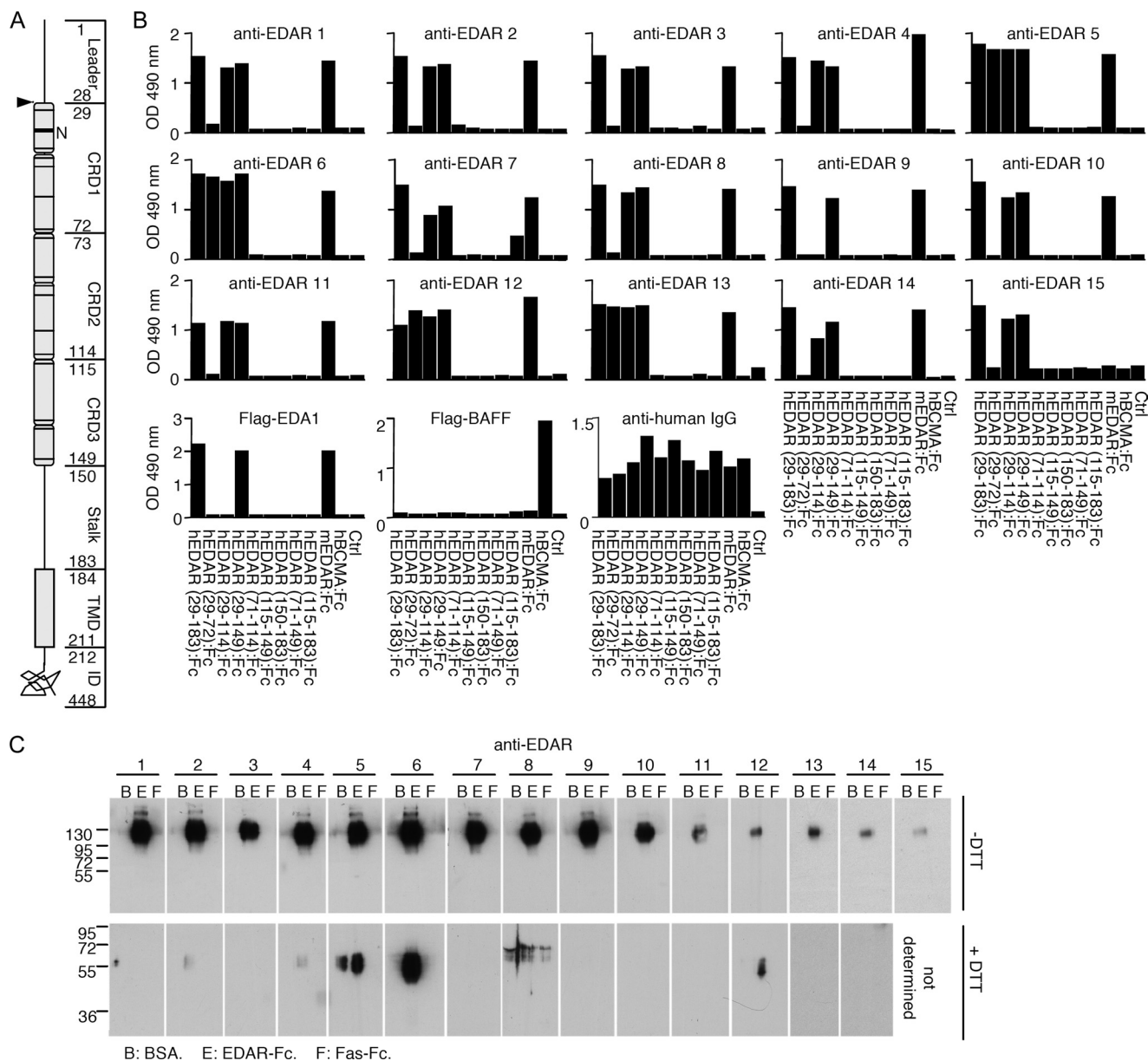


FIGURE 2. Epitope mapping of anti-EDAR monoclonal antibodies. *A*, schematic linear representation of human EDAR showing the position of cysteine residues (thin horizontal lines), the putative *N*-linked glycosylation site (thick horizontal line, N), and the six structural modules (rectangles with rounded corners) composing the three cysteine-rich domains (CRD1, CRD2, and CRD3). The transmembrane domain (TMD), signal peptide (Leader), stalk, and intracellular domain (ID) are also shown. Amino acid numbers at the junctions of interest are indicated. The arrowhead indicates the predicted cleavage site of the signal peptide. The scheme is drawn to scale, except for the intracellular domain. *Ctrl*, control. *B*, the indicated EDAR-Fc constructs or B cell maturation antigen-Fc (BCMA-Fc) control were captured in an ELISA plate and revealed with the indicated anti-EDAR antibodies, with an anti-human IgG to control efficient capture of the various EDAR-Fc proteins, or with FLAG-EDA1 or FLAG-BAFF as controls. *C*, bovine serum albumin (B; BSA, 2 μ g), hEDAR-Fc (E, 20 ng), and hFas-Fc (F, 20 ng) were resolved by SDS-PAGE under reducing or nonreducing conditions, transferred onto nitrocellulose, and probed with anti-EDAR1–15.

dysplasia were administered agonist anti-EDAR antibody 3 in the jugular vein at 2 days of life ($n = 2$; 10 mg/kg) or at 14 days of life ($n = 1$; 7 mg/kg). The analysis was performed essentially as described previously (17, 25). In particular, the dogs were monitored daily for adverse reactions, overall health, and specific ocular and respiratory diseases. Complete blood cell counts and serum biochemistry screens were evaluated within 2–7 days after injection with agonist anti-EDAR antibody. Dental radiographs were obtained when the dogs were adults, when about 1 year old. Shirmer tear testing was performed at 6-month intervals. Complete necropsies were performed between 1.6 and 2.4 years of age. Tissues were fixed in 10%

neutral buffered formalin, routinely processed, sectioned at 5 μ m, and stained with hematoxylin and eosin.

RESULTS

Generation and Screening of Agonist Anti-EDAR Antibodies—To obtain cross-reacting antibodies against conserved EDAR regions, EDAR-deficient mice were immunized with either human or mouse EDAR-Fc fusion proteins. The EDAR-deficient mouse strains *Downless* and *Sleek* have loss-of-function mutations in the extracellular or intracellular domains of EDAR, respectively, but still express the protein. We therefore immunized OVE1B mice in which the *Edar* gene is completely

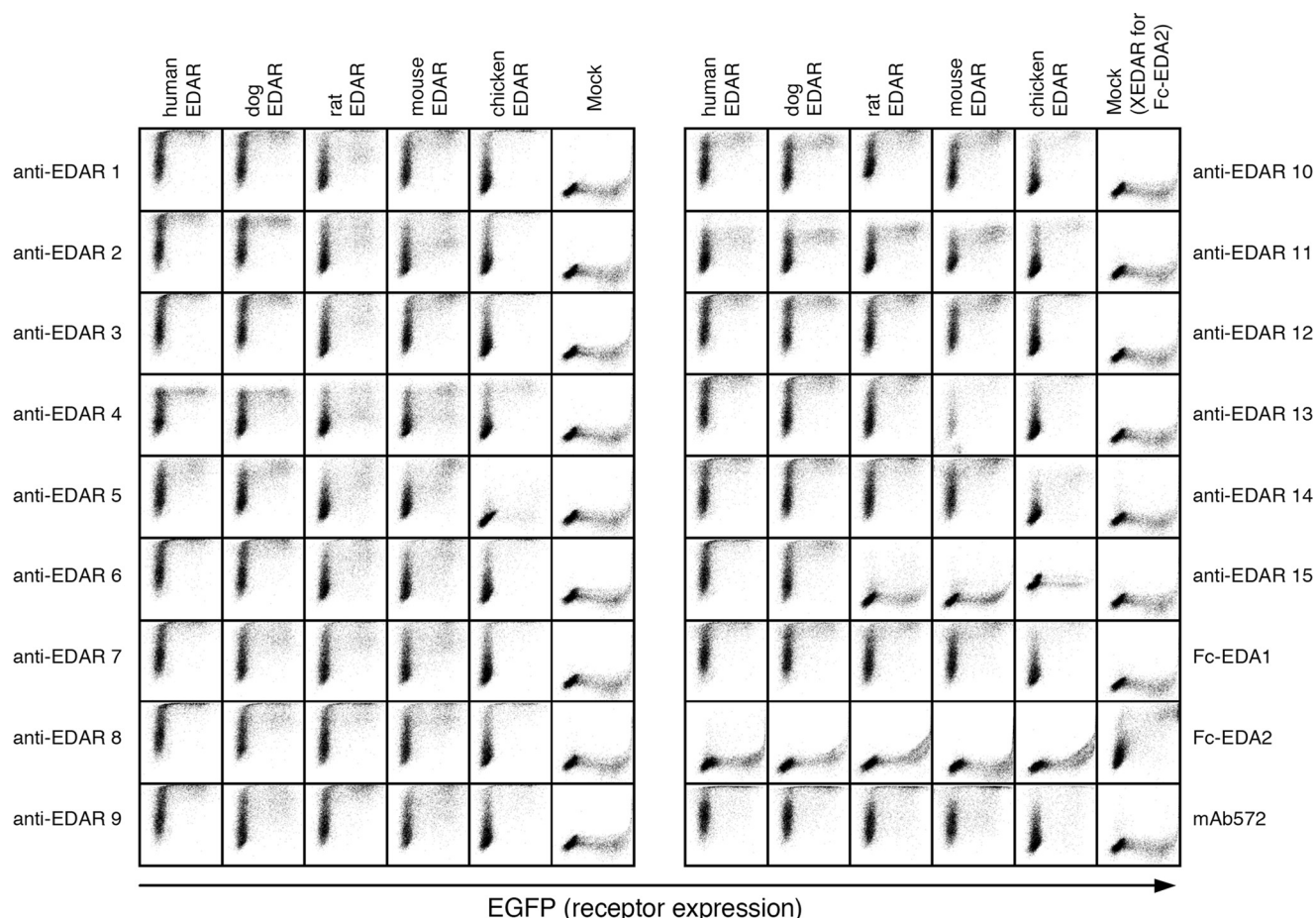


FIGURE 3. Cross-species specificity of anti-EDAR antibodies. Receptors fused to the glycosylphosphatidylinositol anchor of TRAILR3 were expressed in 293T cells together with an enhanced GFP tracer (x axis) and stained with Fc-EDA1, Fc-EDA2, or with anti-EDAR antibodies (y axis). Receptor expression was confirmed by staining with an antibody directed against the C-terminal portion of TRAIL-R3 present in all constructs (mAb572). Both scattergram axes show fluorescence intensity on a logarithmic scale (10^0 – 10^4).

deleted by random genomic integration of an unrelated transgene (5, 26). Hybridoma supernatants with anti-EDAR reactivity were screened for agonist activity using two complementary tests. In the first one, surrogate reporter cell lines stably expressing hEDAR:Fas or mEDAR:Fas fusion proteins were used. EDAR activation in these reporter cells leads to apoptotic cell death by activation of the Fas pathway (13). In the second assay, hybridoma supernatants were administered intraperitoneally to newborn, EDA-deficient *Tabby* pups (18). *Tabby* mice completely lack tail hairs, and the agonist activity of antibodies can therefore be measured by the induction of tail hair, provided that antibodies recognize and activate mouse EDAR. Several hybridoma secreted agonist antibodies with *in vivo* activity, but only a few were also active in the cell-based *in vitro* assay (data not shown). Selected hybridoma were subcloned and adapted for growth in serum-free medium from which antibodies were purified (Fig. 1A). The monoclonal nature of these antibodies was further confirmed by their sharp migration in native protein electrophoresis (Fig. 1B).

Agonist Anti-EDAR Antibodies Have Varied but Relatively Limited Sequence Characteristics—Variable regions of heavy and light antibody chains were amplified by RT-PCR and sequenced for several hybridoma. A number of different variable region genes were identified for both the heavy and light

chains (Table 1), but some of them were shared by two or three hybridoma, usually with different somatic mutations. Interestingly, antibodies with identical heavy and light variable genes sharing greater than 90% sequence identity were obtained from different mice immunized with mouse (antibody 8) or human EDAR (antibodies 1 and 3) (Table 1 and supplemental Fig. S2). Thus, different variable genes can be used to generate agonist anti-EDAR antibodies, but the gene repertoire must be limited as similar antibodies were found two or three times in the relatively limited panel that we have analyzed.

Agonist Antibodies Recognize at Least Three Different Epitopes in EDAR—EDAR contains three cysteine-rich domains in its extracellular region, plus a stalk sequence (Fig. 2A). We used EDAR constructs containing these four regions alone or in combinations to roughly characterize the epitopes recognized by the antibodies. Some antibodies recognized CRD1 alone, others recognized CRD1 and -2 together, and one reacted with CRD1–3, but none bound to receptors lacking CRD1 (Fig. 2B and Table 1). All antibodies recognized recombinant EDAR by Western blot under nonreducing conditions, but only three reacted with reduced EDAR (Fig. 2C). These three antibodies had their epitopes localized in CRD1. It is possible that the presence of CRD1 is required for correct folding of EDAR. Abnormal disulfide bridge formation in the absence of

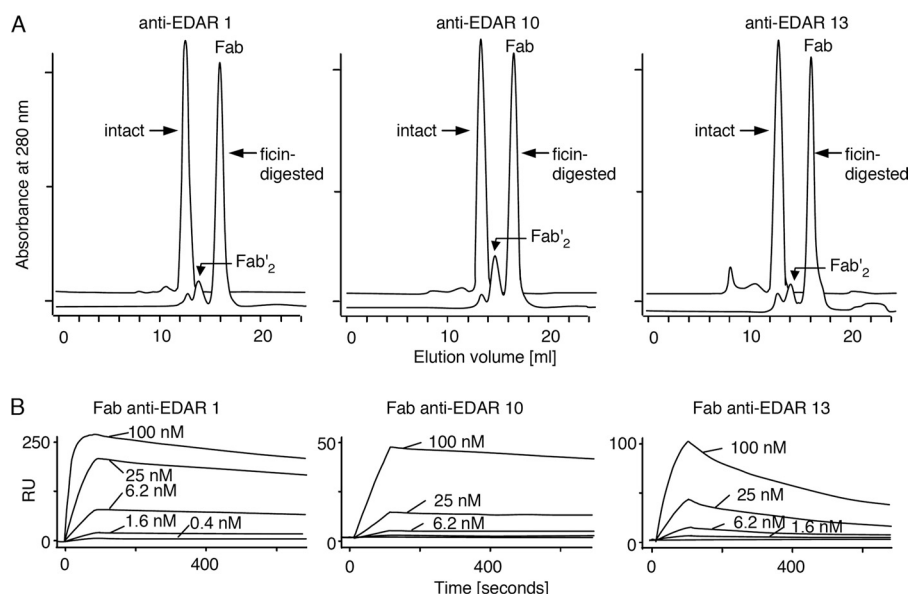


FIGURE 4. Generation and binding characteristics of anti-EDAR Fab fragments. A, Superdex-200 gel permeation chromatography elution profiles of intact and ficin-digested anti-EDAR antibodies. In the digested antibodies, Fc fragments and undigested antibodies were first removed by chromatography on protein A. Peak identities are indicated. B, Fab fragments at the indicated concentrations were analyzed by surface plasmon resonance onto immobilized hEDAR-Fc. Fab solutions were applied for 90 s, and subsequently washed with buffer. Results for three antibodies with distinct binding characteristics are shown.

CRD1 would explain why antibodies did not recognize constructs containing CRD2 and CRD3. Taken together, these results indicate that agonist anti-EDAR antibodies can recognize at least three different EDAR epitopes located in CRD1 and probably CRD2 and CRD3.

Agonist Anti-EDAR Antibodies Cross-react with EDAR of Various Mammals and Birds—Most anti-EDAR antibodies cross-reacted with human, dog, rat, mouse, and chicken EDAR when these were expressed as glycosylphosphatidylinositol-anchored molecules in 293T cells (Fig. 3). Antibody 5 only reacted minimally with chicken EDAR, whereas antibody 15 that had been selected on the basis of its specificity for human EDAR rather than for its agonist activity recognized human and dog EDAR, but not rat, mouse and chicken EDAR (Fig. 3).

Binding Characteristics of Anti-EDAR Antibodies to EDAR—The affinity of 11 agonist antibodies to human EDAR was determined by surface plasmon resonance. For this purpose, monomeric Fab fragments were generated by ficin digestion and size exclusion chromatography (Fig. 4A). Affinities varied from 0.5 to 40 nM, and differences were also observed in the association and dissociation constants (Fig. 4B and Table 1). The dissociation kinetics of two antibodies (7 and 14) were biphasic, showing first a rapid dissociation followed by a slower dissociation, but as these antibodies had no remarkable agonist activity, this was not analyzed further.

Comparison of the Activity of Agonist Anti-EDAR Antibodies *in Vitro* and *in Vivo*—As we have not yet been able to identify a simple and quantitative assay to characterize EDAR agonists using EDAR's own signaling pathway *in vitro*, we used a surrogate reporter assay in which Fas-sensitive cells were transfected with the extracellular domain of human or mouse EDAR fused to the intracellular portion of Fas. Binding of an active recombinant EDA1 (Fc-EDA1) to these cells induces cell death by

activation of the pro-apoptotic Fas pathway (13). Interestingly, only some of the antibodies (1, 3, 8, 10, and 12) (Fig. 5A) killed mEDAR:Fas-expressing cells, and even fewer killed hEDAR:Fas expressing cells (1, 3, 10, and 12) (Fig. 5B and Table 1). In all cases, antibodies were less active than Fc-EDA1 by 1–2 orders of magnitude. The picture was different in an *in vivo* assay, where newborn *Tabby* mice were treated with antibodies on the day of birth. In this case, all antibodies rescued tail hair formation in a dose-dependent manner and with similar EC_{50} values of 0.1 to 0.7 mg/kg (Fig. 6). Only one of the antibodies (antibody 11) seemed less active, with an EC_{50} value of about 3 mg/kg. Functional sweat glands were induced by the treatment with similar EC_{50} as for tail hair (data not shown). The half-life of two antibodies (1 and 3) was determined in adult *Tabby* mice and found to be 10.5 and 11 days, respectively (supplemental Fig. S3).

Divalent Monomeric Agonist Antibodies Are Active *in Vivo*—We have shown previously that cross-linked EDA1 containing more than one trimer in a single molecule are better agonists than trimeric EDA1 (13). We therefore wondered whether the *in vivo* activity of agonist anti-EDAR antibodies was due to monomeric antibodies or to aggregates thereof. The activity of a monomeric (divalent) antibody purified by size exclusion chromatography (Fig. 6, antibody 1) was, however, very similar to that of the total preparation (data not shown), and compared favorably (EC_{50} ~0.1 mg/kg) with recombinant Fc-EDA1 (EC_{50} ~0.05 mg/kg) (Fig. 6). In addition, the $F(ab')_2$ fragment, but not the Fab fragment, was active *in vivo* when administered to newborn pups on the day of birth (supplemental Fig. S4). When the $F(ab')_2$ was administered later at day 3 post-birth, one of the latest time points where ventral tail hair can be induced, its action could be inhibited by an excess of the Fab fragment, ruling out that the lack of agonist activity of the Fab would be due only to a shorter half-life *in vivo* (supplemental Fig. S4). We

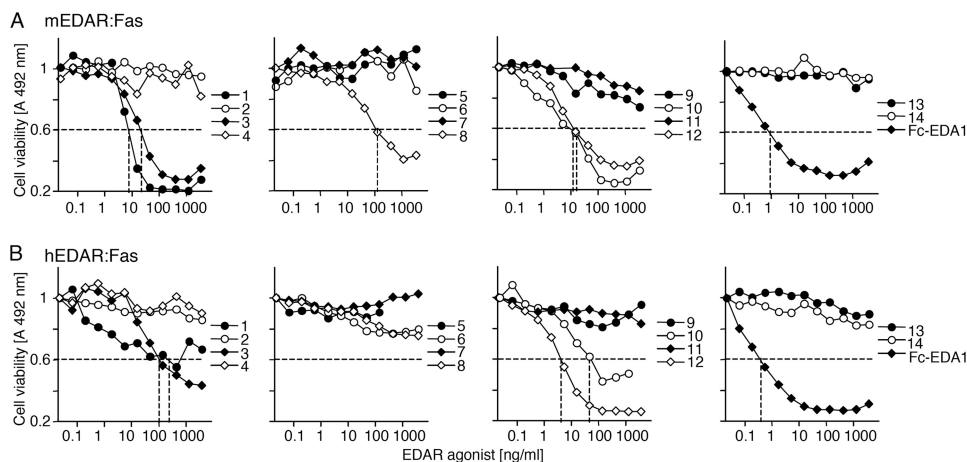


FIGURE 5. *In vitro* activity of anti-EDAR antibodies in a surrogate reporter assay. *A*, anti-EDAR antibodies and Fc-EDA1 were tested for their capacity to induce apoptosis of mEDAR:Fas-expressing Jurkat cells. After overnight culture, cell viability was determined by a cell viability assay. EC_{50} are indicated as dotted lines. Anti-EDAR antibody number (or Fc-EDA1) for each curve is indicated to the right of each panel. *B*, as above, except that hEDAR:Fas expressing cells were used.

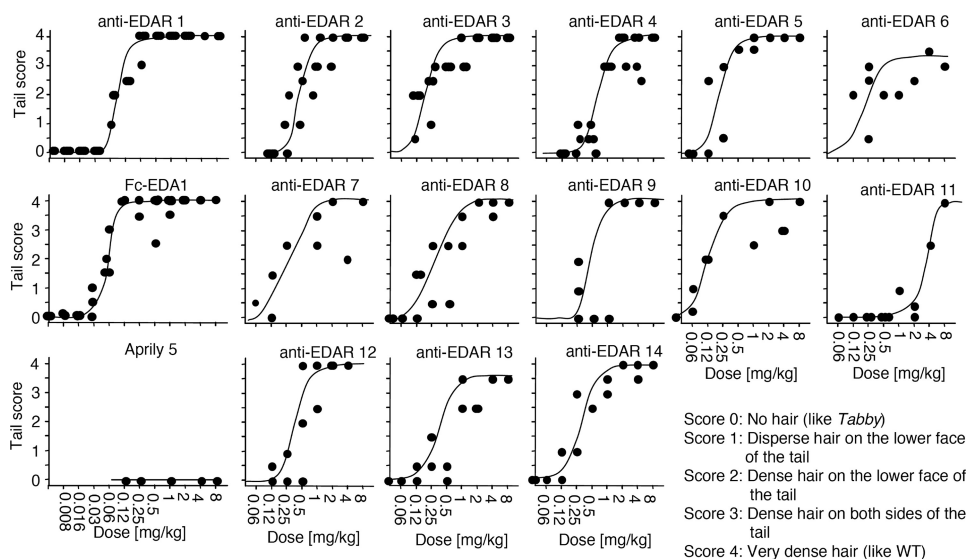


FIGURE 6. Therapeutic doses of the anti-EDAR antibodies in newborn *Tabby* mice. Newborn *Tabby* mice were injected intraperitoneally during the first 24 h of life with graded doses of anti-EDAR antibodies, an irrelevant mouse IgG1 (April 5) or Fc-EDA1. Four to six weeks later (antibody 2–4) or 3 weeks later (antibodies 1, 4–14, and April 5), hair density on the tail was scored according to the criteria shown in the inset. The anti-EDAR antibody 1 used in this experiment was the monomer peak obtained by gel filtration (see Fig. 4).

conclude from these observations that a divalent agonist anti-EDAR antibody is both sufficient and necessary to exert activity in *Tabby* mice.

Effective Treatment of EDA-deficient Mice with Agonist Anti-EDAR Antibodies—Some patients with partially inactivating EDA mutations have teeth defects but otherwise normal skin appendages, suggesting that tooth formation may require more stringent EDAR signals than other skin appendages for proper development (14). To test the effect of agonist antibodies on tooth development, pregnant *Tabby* mothers were treated during pregnancy so that the antibody could be transferred to embryos by the trans-placental antibody transport system. Mice exposed to agonist anti-EDAR during development not only had tail hairs and functional sweat glands, but also hair behind the ears, mucus-secreting glands in the trachea, and a normalized eye appearance (Fig. 7, A–G, and supplemental Fig. S5). In addition, molars of treated mice were reverted and

almost indistinguishable from those of wild type animals (Fig. 7, H and I, and supplemental Fig. S5). The effect was long lasting, as a similarly treated mouse was still reverted after more than 2 years (supplemental Fig. S6). Taken together, these results indicate that the two agonists anti-EDAR antibodies tested in this application (antibodies 1 and 3, Fig. 7, supplemental Fig. S4, and data not shown) revert the ectodermal dysplasia phenotypes that we have looked at in *Tabby* mice, including tooth morphology.

Activity of Agonist Anti-EDAR Antibodies in EDA-deficient Dogs—Agonist anti-EDAR antibodies recognize EDAR of different species (Fig. 3). To test whether the observed cross-species reactivity also holds true for the agonist activity, anti-EDAR antibody 3 was administered intravenously to three EDA-deficient dogs at either 2 days of life ($n = 2$, 10 mg/kg) or at 14 days of life ($n = 1$, 7 mg/kg). None of these dogs showed adverse reactions upon injection. Dentition was corrected not only in

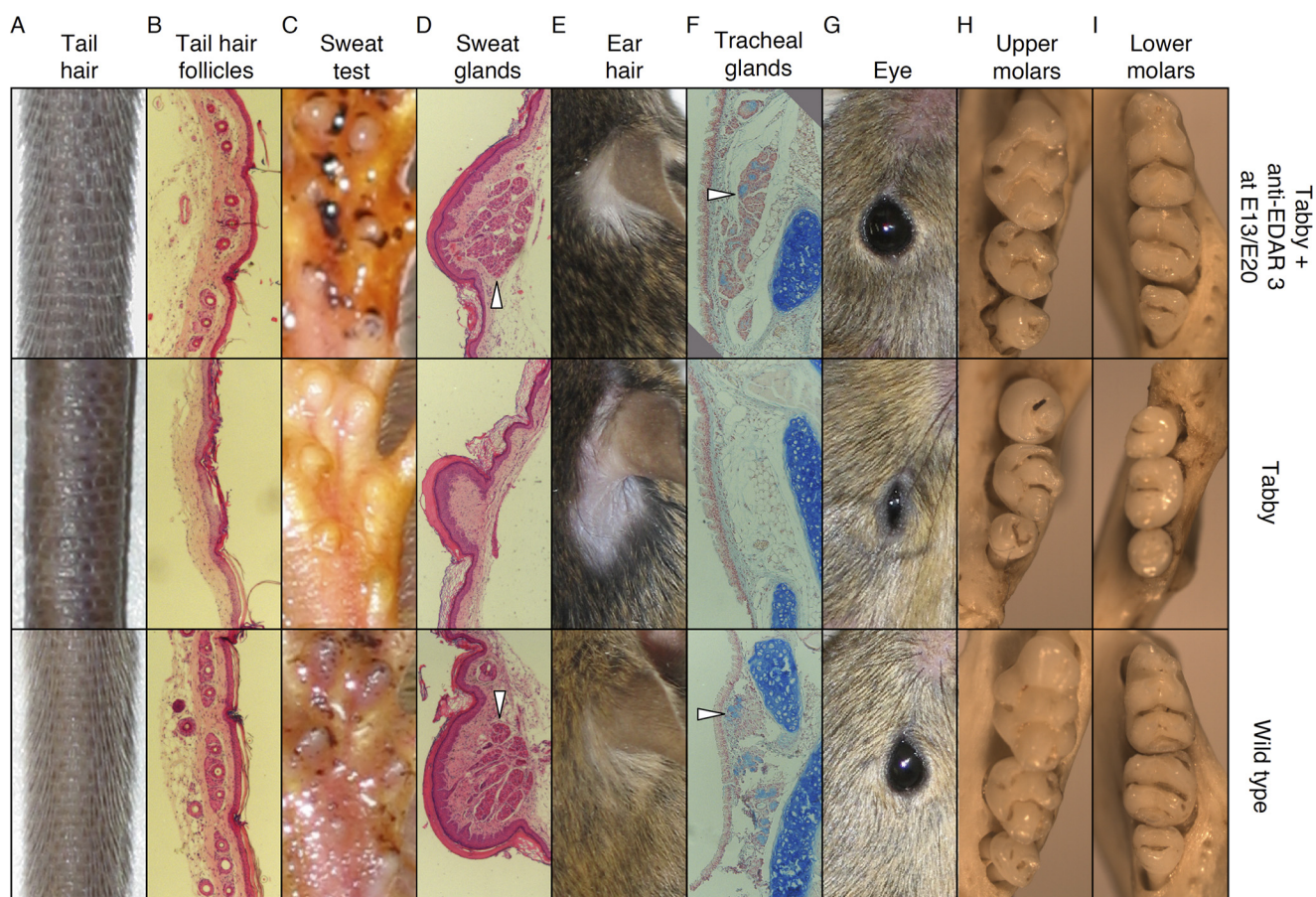


FIGURE 7. Anti-EDAR antibody reverts many ectodermal dysplasia phenotypes in EDA-deficient mice. Pregnant *Tabby* mice were treated intravenously at days 13 and 20 (E13/E20) of gestation with anti-EDAR antibody 3 at 16 mg/kg. Offspring were analyzed at 6 months of age. Age-matched wild type and EDA-deficient *Tabby* mice were similarly analyzed for comparison. *A*, tail phenotype. *B*, transversal sections of tail skin showing the presence of hair follicles, stained with hematoxylin and eosin. *C*, starch iodine sweat tests. *D*, sections of foot pads showing the presence of glandular structures of sweat glands (arrowheads), stained with hematoxylin and eosin. *E*, top view of the retro-auricular region showing the presence of hair behind the ears. *F*, sections of the trachea stained with Alcian blue to reveal mucus-secreting glands (arrowheads). *G*, eye phenotype showing reversion of the thickened eyelid margin and narrow eyelid opening. *H* and *I*, pictures of the jaw carrying the upper and lower molars. Antibody treated and wild type jaws carry larger teeth with a normal pattern of cusps on their surfaces.

EDA-deficient dogs that were treated at 2 days of life but also in the affected treated at 14 days of life, although the latter still lacked premolars, accounting for the decreased number of teeth (Fig. 8 and Table 2). Interestingly, the premolars and molars in dogs treated at day 2 of life had a more normal appearance than in those dogs treated with Fc-EDA (17). Lacrimation was improved in treated dogs except in one treated at day 2 of age (Table 2). The correction of glands in trachea, bronchi, and esophagus appeared, however, to be dependent on the age at which the dogs were treated, *i.e.* treatment administered earlier in life had a bigger impact on gland development (Fig. 8 and Table 2). It is noteworthy that, regardless of the extent of the phenotypic reversion, none of the treated dogs suffered from pneumonia or other airway diseases that are common in untreated EDA-deficient dogs or from dry eye condition (keratoconjunctivitis sicca) that affect all XLHED dogs.

DISCUSSION

Deficiency in the TNF family ligand EDA leads to ectodermal dysplasia, even if the receptor EDAR remains fully functional. The development of EDAR agonists are thus of inter-

est for applications in the treatment of XLHED. EDAR is relatively well conserved across species, with only 4 amino acid differences between human and dog, 10 between human and mouse, and 13 between human and chicken in the 154-amino acid-long mature extracellular domain (supplemental Fig. S7). To increase the likelihood of getting cross-reactive antibodies, EDAR-deficient mice were used to generate monoclonal antibodies. The approach proved successful as 13 of the 15 anti-EDAR antibodies analyzed recognized EDAR from human, dog, rat, mouse, and chicken. These antibodies are therefore likely to cross-react with EDARs of all mammals and many other vertebrates. Anti-EDAR15 differentiated human and dog from rat and mouse EDARs whose primary sequence only diverge in CRD1 (supplemental Fig. S7), implying that part of its epitope is in CRD1. Anti-EDAR9 recognized CRD1–3 of EDAR but none of the cysteine-rich domains taken individually or in pairs, despite the fact that these fragments were overlapping and supposed to respect structural elements of the receptor. These results indicate that EDAR may fold on itself to create a conformational epitope with regions that are distant in the primary

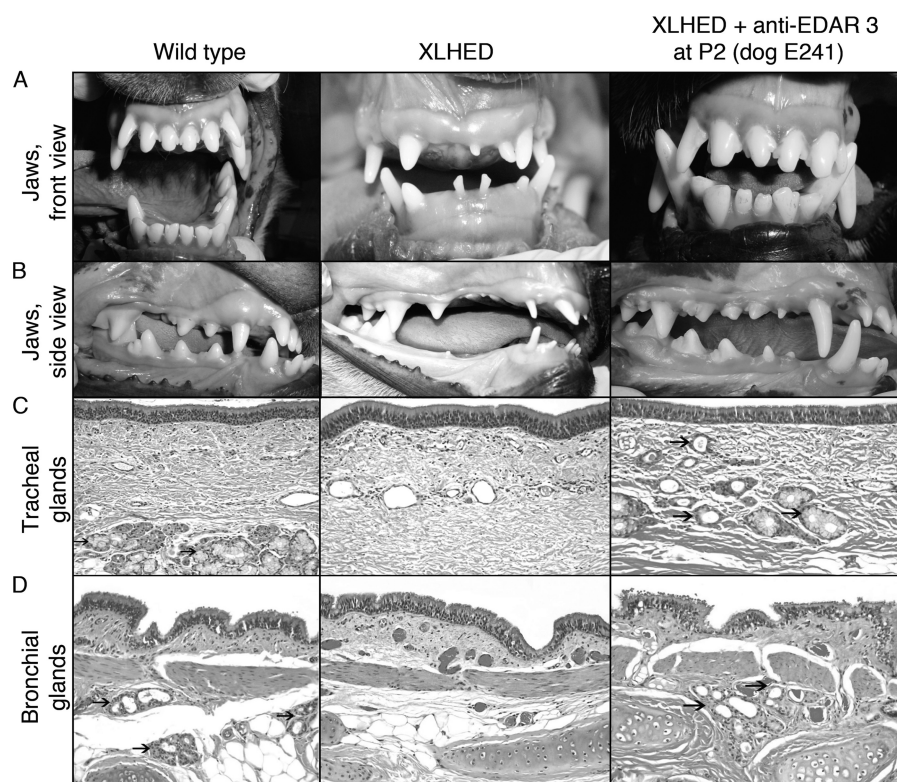


FIGURE 8. **Anti-EDAR antibody ameliorates dentition and presence of glands in EDA-deficient dogs.** An EDA-deficient dog was treated at day 2 of life with a single dose of anti-EDAR (antibody 3) at 10 mg/kg, and analyzed 1.6 years later in comparison with a wild type and with an affected dog. *A* and *B*, front and side views of the jaws. *C* and *D*, hematoxylin and eosin-stained tissue sections of the trachea and bronchi. Glandular tissues are indicated with arrowheads.

TABLE 2

Summary of clinical and pathological findings in untreated XLHED dogs and XLHED dogs treated with anti-EDAR antibody 3

Dog number	Treatment protocol	Age at necropsy	Appearance of teeth (number)	Tracheal glands	Bronchial glands	Esophageal glands	Tear production %
Wild type (<i>n</i> = 5)		1–3 years	Normal (41.6 ± 0.9)	+++	+++	+++	97 ± 14
XLHED (<i>n</i> = 6)		1–3 years	Abnormal (18.0 ± 2.9)	None	None	None	68 ± 20
E237	10 mg/kg on day 2	1.7 years	Greatly improved (40)	+++	+++	+++	70.0 ± 4.1
E241	10 mg/kg on day 2	1.6 years	Greatly improved (41)	++	+	++	90.0 ± 7.1
E222	7 mg/kg on day 14	2.4 years	Improved (30)	+	–	+	80.0 ± 27.1

sequence. In any case, results indicate that agonist anti-EDAR antibodies can recognize at least three distinct epitopes in EDAR.

Anti-EDAR antibodies were screened in newborn *Tabby* mice, a highly relevant *in vivo* assay whose main limitation is to detect only antibodies cross-reacting with mouse EDAR. More than half of the 46 hybridoma supernatants tested were active in this assay, indicating that agonist antibodies can readily be obtained with the procedure used. The success rate of detection was lower using the EDAR:Fas reporter cell lines that signal cell death in an oligomerization-sensitive manner, probably because these cell-based assays make use of a different intracellular signaling pathway. When combined, these assays discriminated two classes of agonist anti-EDAR antibodies, with or without *in vitro* activity. Interestingly, the *in vitro* activity correlated relatively well with low antibody dissociation constants but not with association constants or affinities (supplemental Fig. S8). The EDAR:Fas reporter cells were previously shown to discriminate recombinant WT EDA1 and EDA1 with the

V365A mutation identified in a family with selective tooth agenesis, despite the fact that these two ligands bind EDAR-Fc almost equally well (14). This led to the hypothesis that formation of teeth may require higher quality EDAR signals than those needed for hair or gland formation. Thus, it is possible that agonist antibodies with activity *in vitro* may also be the best ones to correct tooth defects associated with EDA deficiency. It will be interesting to experimentally test this hypothesis in the future.

The plethora of anti-EDAR agonist antibodies obtained, most of which were of the IgG1 isotype, was surprising. Indeed, there are indications that agonist anti-Fas antibodies (Fas is another TNFR family member) need to be oligomerized to be active. For example, the CH11 IgM monoclonal antibody directed against human Fas was obtained by immunization of mice with membranes of FS-7 human fibroblasts (27). When an IgG1 recognizing the exact same epitope (mAb ZB4) or a divalent F(ab')₂ of CH11 was used, there was no agonist activity (28). A second example of an agonist

monoclonal antibody directed against human Fas is APO-1, which was obtained by immunizing mice with plasma membranes of the human SKW 6.4 cell line (29). This antibody is an IgG3. Upon isotype switch, it loses its agonist activity (30), a result that was tentatively explained by the propensity of IgG3 to self-aggregate. As immunoglobulin preparations often contain low amounts of high molecular weight antibody aggregates (31), we wondered whether the agonist activity of anti-EDAR antibodies could be due to aggregates. This was not the case, however, as monomeric antibodies had a similar specific activity *in vivo* as the total preparation. In addition, a purified F(ab')₂ fragment was agonist *in vivo*, whereas the Fab fragment was not. We conclude that divalency is necessary and sufficient for anti-EDAR agonist antibodies to exert their activity.

We have shown previously that the collagen domain of EDA oligomerizes the trimeric TNF homology domain of EDA1 into higher order structures with concomitant gain of activity (13). Similarly, Fc-EDA1 that assembles as a hexamer (containing two EDA1 trimers) is a highly active molecule. One interpretation is that multiple EDAR molecules must be recruited within the same complex to deliver robust intracellular signals, but this is in apparent contradiction with the observation that divalent antibodies are good agonists. A first hypothesis to reconcile these observations is that EDAR may pre-exist as inactive complexes before ligand binding, as shown previously for Fas (32). Binding of a ligand may change the conformation of the complex to render it signaling-competent and bring together two such complexes to initiate signaling. Divalent agonist antibodies may mimic hexavalent ligands by inducing the conformational change by binding one receptor in the pre-assembled complex and recruiting and activating a second complex on its second arm. In a second hypothesis, binding of the ligand or the antibody at an appropriate site of EDAR is sufficient to render the receptor signaling-competent. Assembly of the signaling complex may be relatively slow and reversible if the agonist detaches prematurely from the receptor. In this model, efficient signaling could be obtained either with divalent reagents with low dissociation constants or with ligands that compensate relatively high dissociation constants by multivalency. Whatever their mechanism of action, Fc-EDA1 and agonist anti-EDAR antibodies are in practice excellent agonists to cure animal models of XLHED, including their teeth defects. Because of their long half-life *in vivo*, agonist anti-EDAR antibodies will prove useful reagents for long term experiments, especially in mice where these mouse antibodies should elicit minimal neutralizing immune responses.

Finally, it is noteworthy that keratoconjunctivitis sicca (dry eye) that affects all untreated EDA-deficient dogs is believed to be caused by decreased tear production. Tear production improved significantly in two of the treated dogs but not in a third one (E237). Nevertheless, none of the dogs treated in this study with agonist anti-EDAR antibody required therapy for dry eye or any other ocular disorders. These findings suggest that decreased tear production is not the only factor causing dry eyes but that other structures such as Meibomian glands that

lubricate the eye, or the composition of the lipids therein, also play an important role.

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